to the pathogenesis of atherosclerosis, including *C. pneumoniae*[3] and *Helicobacter pylori*[22]. Such bacteria may cause vascular injury by direct colonization[23] or by activation of a systemic inflammatory response, which may play a role in the progression and destabilization of atherosclerotic plaques. In support of this view, we found a higher incidence of death in patients with *C. pneumoniae* infection than in the uninfected patients, but the difference was not statistically significant[8]. A new study with a longer observation period will be required to determine the precise relationship between *C. pneumoniae* infection and CAD, as well as why C. pneumoniae infection is associated with a resistance of carotid atherosclerosis to lipid-lowering therapy.

The following possible mechanisms may explain the relationship between HCV infection and atherosclerosis. First, HCV infection is occasionally associated with vasculitis[24]. Second, chronic HCV infection may be associated with an increase of oxidative stress[25]. Third, HCV infection can produce elements of the metabolic syndrome by inducing insulin resistance[26], which may accelerate atherogenesis. Finally, chronic HCV infection may stimulate a systemic inflammatory response. Another study with a longer observation period will be required to determine the precise relationship between HCV infection and atherosclerosis, as well as why HCV infection was associated with the effect of lipid-lowering therapy on carotid atherosclerosis.

Evidence from epidemiological and clinical studies has shown that LDL cholesterol is very important in the development of atherosclerosis and that reducing the LDL cholesterol level can also reduce the risk of CAD. We previously reported that active treatment may not only have a lipid-lowering effect but may also stabilize plaque and in the same study demonstrated a lower incidence of cardiac events in the treated group than in the control group[3]. In the present study, we found a significant reduction of the serum total and LDL cholesterol levels in both HCV-infected and uninfected patients. Although total and LDL cholesterol levels were reduced in the HCV-infected patients by active treatment, the carotid IMT was not improved as occurred in the uninfected patients. The above finding suggests that HCV infection is a risk factors for progression of atherosclerosis. If it possible that the organism may be an innocent bystander in atherosclerotic tissue, rather than an inciter of chronic inflammation. Determining the exact nature of the association between HCV and atherosclerosis is important. The above suggests that HCV was found to be a causative factor or to significantly contribute to the progression of atherosclerosis, so it might be improved by the interferon therapy which is able to eliminates HCV RNA in patients with chronic HCV infection[27][28].

The present study had some limitations. First, the subjects were only defined by their serological parameters and not by histology. However, histological examination is more suitable for a clinical setting than for population-based studies. Second, the duration of exposure to viral infection could not be estimated because the detection of antibodies was usually incidental. Thus, the duration of inflammation may have been too short and the infection too mild to detect any pro-atherosclerotic effect in some of the subjects. Although care was taken to avoid potential biases, it is well known that prospective studies are often

unable to confirm the associations detected by case-control studies. Therefore, a future prospective study is needed to confirm our findings.

In conclusion, our observations suggest that both HCV infection and *C. pneumoniae* infection reduce the efficacy of lipid-lowering therapy for carotid atherosclerosis, and that HCV may play a role in the development/progression of atherosclerosis.

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(和文抄録)

脂質低下療法による動脈硬化症の進展抑制における C型肝炎ウイルス及肺炎クラミジア感染の影響

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【目的】 慢性感染症は冠動脈疾患の危険因子の一つとされており,私どもは以前肺炎クラミジア感染に関して報告した. 今回, C型肝炎ウイルス感染が独立して脂質低下療法による動脈硬化症の進展抑制に影響を及ぼすかどうかについて検討した.

【方法】 対象は高コレステロール (TC) 血症患者165例 (30歳～89歳・平均年齢64歳, 男性45例, 女性120例)を対象とし, 抗高脂血症剤(プロブコールあるいはプラバスタチン) を投与し, 血清脂質の測定およびBモード超音波法による総頚動脈の内膜中膜複合体厚 (IMT) の測定を施行し, 2年間経過観察した. HA抗体(ELISA法), HBs抗原(ELISA法), HCV RNA(PCR法), 肺炎クラミジア-IgA, IgG抗体 (ELISA法) で測定した.

【結果】 血清脂質値は, 脂質低下療法にてHAV, HBV及びHCVの感染の有無にかかわらず, いずれも有意な減少を認めた. Max-IMT値の減少率は, HAV及びHBVの感染の有無にかかわらず有意差はみられなかったが, HCV感染陰性の場合は, 感染陽性の場合より脂質低下療法による動脈硬化の進展抑制効果が有意に認められた($P<0.05$). さらに多変量解析にて, Max-IMT変化率に寄与する因子を検討したところ,肺炎クラミジア感染及びHCV感染は治療効果の独立した負の因子であった.またプロブコールとプラバスタチンの間では感染の有無および治療効果に差はみられなかった.

【考察】 肺炎クラミジア感染だけでなく, HCVの持続感染も,脂質低下療法による動脈硬化症の進展抑制効果を妨げる可能性が示唆された.

Original Article

Efficacy of Intravenous Glycyrrhizin for the Treatment of Chronic Hepatitis C : A Comparison of the Original and Generic Drugs

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Original Article

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ABSTRACT : The utilization of generic drugs in medical practice has been promoted in Japan for the purpose of minimizing drug costs. In order to determine the clinical efficacy of the original preparation of glycyrrhizin, in comparison to its generic drug, a controlled longitudinal study was done of 82 consecutive patients with chronic hepatitis C receiving the original preparation of glycyrrhizin for 6 months. Patients treated with the original preparation of glycyrrhizin for 6 months at two hospitals were separated into two groups for study : Patients who changed from the original preparation of glycyrrhizin to a generic drug and then changed back from the generic drug to the original preparation of glycyrrhizin (Group A, n=46) ; and, patients who were continuously treated with the original preparation of glycyrrhizin (Group B, n=36). HCV RNA levels were serially determined by Cobas Amplicor HCV Monitor assay. In Group A, the ALT level significantly elevated 3 months after switching treatment from the original preparation of glycyrrhizin to the generic drug (from 65.1±22.7 IU/L to 112.4±39.9 IU/L) ($P<0.05$), then significantly decreased 3 months after the change back to the original preparation of glycyrrhizin (from 112.4±39.9 IU/L to 62.1±23.0 IU/L) ($P<0.05$). In Group B, however, the ALT level did not significantly change during the same observation period. The serum HCV RNA level did not significantly change in either group, even in Group A patients whose ALT levels significantly changed. The efficacy on ALT of the original preparation of glycyrrhizin and the generic drugs differed in patients with chronic hepatitis C.

KEY WORDS : glycyrrhizin, chronic hepatitis C, generic drug

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Chronic hepatitis C virus (HCV) infection has become the most frequent cause of chronic liver disease worldwide. Population based surveys in Japan have reported the prevalence of HCV to be 0.7-3.7%.[1-3] Chronic HCV viremia often follows a progressive course over many years and can ultimately result in cirrhosis and hepatocellular carcinoma (HCC).[4,5] Over the past decade, more than 80% of Japanese patients with HCC were found to have chronic HCV viremia.[6,7] The risk of HCC development

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has been reported to be higher in HCV-infected patients who have biochemically and histologically active chronic hepatitis, suggesting that necroinflammation and its associated regenerative processes play a pivotal role in hepatic carcinogenesis.[8] Interferon (IFN) has been shown to eliminate HCV viremia and to reduce serum alanine aminotransferase (ALT).[9-11] However, IFN is not effective for all patients, and is sometimes not possible because of its high cost and side effects. Stronger Neo-Minophagen C® (SNMC), a preparation of glycyrrhizin, is a well-known Japanese medicine that is commonly administered to improve the serum ALT level of chronic hepatitis C patients, especially those resistant to IFN or who relapse after IFN treatment.[12]

SNMC is used for the treatment of allergic diseases and hepatitis in Japan. In 1977, intravenous injection with SNMC was permitted for patients with chronic hepatitis or liver cirrhosis, most of whom were infected with hepatitis viruses.[13] SNMC is reported to be effective for the reduction of elevated ALT levels in patients with chronic hepatitis B[14] and chronic hepatitis C.[15] Recently, van Rossum, TG and colleagues have done serial clinical studies on the pharmacokinetics, effectiveness, and adverse effects in European patients with chronic hepatitis C receiving SNMC.[16-19]

A generic drug is identical, or bioequivalent to a brand name drug in dosage form, safety, strength, route of administration, quality, performance characteristics, and intended use. Although generic drugs are chemically identical to their branded counterparts, they are typically sold at substantial discounts from the branded price. The utilization of generic drugs in medical practice has been promoted in Japan for the purpose of minimizing drug costs ; however, the use of generic drugs has not increased as much as expected. Reasons for this may include concerns about the insufficiency of the information provided on generic drugs, especially about the clinical efficacy.

To determine the efficacy of SNMC (a preparation of glycyrrhizin) and generic drugs, we did a controlled, longitudinal study of Japanese patients with chronic hepatitis C treated with these drugs.

METHODS

Patients

A prospective, controlled study of Japanese patients with chronic hepatitis C was done from 2001 to 2002 in which the efficacy and safety of a single generic glycyrrhizin based drug was accurately compared with SNMC. In the present study, we analyzed the changes of ALT levels and HCV viremic level and the differences of the safety and glycyrrhizin concentration. All 82 patients enrolled in study (43 men and 39 women ; mean±SD age of 63.6±6.0 years ; age range 37-70 years ; serum mean±SD ALT levels at the start of SNMC treatment, 149.9±48.9 IU/L) were seen during routine visits to Kyushu University Hospital, Fukuoka, and Mitsutake Internal Medicine and Circulatory Disease Hospital, Iki Island, Nagasaki, Japan. All patients had serum ALT levels at least 2 times the upper limit of normal (ULN, 35 IU/L) for 12 weeks before SNMC treatment. The SNMC treatment was started for each patient with the aim of normalizing persistently high ALT to reduce the progression of liver disease. After receiving SNMC treatment for 6 months, patients were allocated to the treatment group of their choice : Group A, 46 patients receiving the switching treatment from SNMC to a generic drug, then returning to treatment with SNMC ; and, Group B, 36 patients receiving continuous SNMC treatment. The present study was not randomized, but controlled between the patients of the two hospitals. The Group A patients were followed at Mitsutake Internal Medicine and Circulatory Disease Hospital and the GroupB patients at Kyushu University Hospital. Patient characteristics at the start of the initial SNMC treatment are given in **Table 1**. About 70% of the patients were prior IFN treatment non-responders. No significant differences in characteristics were observed between Group A and B patients at baseline, including height and weight. All patients were positive for serum antibody to HCV and HCV RNA for over 6 months. No patients positive for serum hepatitis B virus surface antigen or antibody to human immunodeficiency virus or having other possible causes of hepatocellular injury, such as autoimmunity or drug-induced liver disease, were included. No patients had received antiviral or corticosteriod therapy within the 12 months prior to inclusion. Needle biopsy of the liver was done for each patient within 6 months before the start of treatment, and two pathologists examined the biopsy specimens independently without previous knowledge of the patients. Using the histological classification by Desmet, et al.[20] and the most widely used "histological activity index" (HAI) by Knodell, et al.,[21] minimal chronic hepatitis (CH) (a score from 1-3 that summed periportal necrosis, intralobular degeneration, and portal inflammation of HAI) was diagnosed in no Group

Table 1. Patient characteristics at baseline

Characteristics	Group A (N=46)	Group B (N=36)	P value
Male N (%)	24 (52.2)	19 (52.8)	NS
Mean body mass index (kg/m²)	23.1	22.9	NS
Mean age (yrs)	63.3	64.6	NS
Prior interferon N (%)	32 (69.6)	25 (69.4)	NS
HCV RNA (100 kIU/ml)	953±246	1156±289	NS
Genotype 1b N (%)	41 (89.1)	31 (86.1)	NS
Cirrhosis N (%)	12 (26.1)	10 (27.8)	NS
Mean ALT level (IU/L)	152.8	150.6	NS
ALT within 2 times ULN N (%)	0−	0−	NS

HCV, hepatitis C virus ; ALT, alanine aminotransferase ; ULN, upper limit of normal (35 IU/L) ; NS, not significant

Baseline is at the start of Stronger-Neo-Minophargen-C treatment.

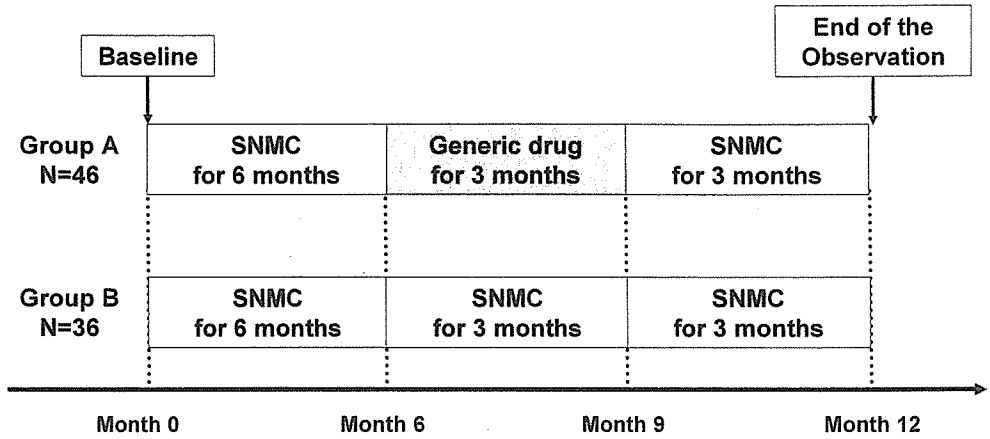


Figure 1. Intervention and study timeline of Group A and B patients. SNMC, Stronger Neo-Minophagen C. Baseline is at the start of SNMC treatment.

A and no Group B patients ; mild CH (a score of 4-8) in 12 Group A and 9 Group B patients ; moderate CH (a score of 9-12) in 11 Group A and 9 Group patients ; severe CH (a score of 13-18) in 11 Group A and 7 Group patients ; and, histologically proven liver cirrhosis (a staging (fibrosis) score of 4 on HAI) in 12 Group A and 10 Group B patients. No significant differences in histological findings were observed between Group A and B patients at baseline. Changes in serum ALT, HCV RNA, the potassium level, and the blood pressure of each patient were measured during the observation period. All patients gave written informed consent before the enrolment of this study. The study was approved by the ethics committee of each hospital. All procedures in the present study were done in accordance with the Helsinki Declaration of 1964 (1996 amended version).

Treatment protocol

Figure 1 shows the intervention and study timelines. The patients of both groups were initially given 60 mL SNMC (Stronger Neo-Minophagen C® , Minophagen Pharmaceutical Co., Ltd, Tokyo, Japan) intravenously three times per week for 6 months (from Month 0 to Month 6). At Month 6, the patients were allocated to the following treatment groups : Group A, 46 who received the switching treatment of the generic drug (Neophagen® , TAIHO Pharmaceutical Co., Ltd, Tokyo, Japan) from Month 6 to Month 9, and then returned for 3 more months of SNMC administration, from Month 9 to Month 12 ; and, Group B, 36 receiving continuous SNMC treatment for 6 months from Month 6 to Month 12. The weekly dosage of each patient was not changed during the observation period.

The package inserts of SNMC and the other generic drugs report that each drug containins 2 mg of

glycyrrhizin, 1 mg of cysteine, and 20 mg of glycine per mL of saline. Neophagen, has the best sales volume of the generic glycyrrhizin drugs sold in Japan.

Serum assay methods

Serum samples were drawn during the observation period and stored at −20°C. They were frozen and thawed only once before doing the qualitative analysis of HCV RNA.

HCV RNA was extracted from 50 μL of serum by Sep Gene RV (Sanko Junyaku, Tokyo, Japan). Complementary DNA was synthesized by the use of random primers and reverse transcriptase (Super Script II ; Life Technologies, Gaithersburg, MD, USA). HCV RNA was detected by 2-stage PCR with primers from the 5' non-coding region of the HCV genome[21] :
5'-CTGTGAGGAACTACTGTCTT-3' (sense) ;
5'-AACACTACTCGGCTAGCAGT-3' (antisense)
in the first stage ;
5'-TTCACGCAGAAAGCGTCTGT-3' (sense) ;
and,
5'-GTTGATCCAAGAAAGGACCC-3' (antisense)
in the second stage.

The HCV RNA genotype of each patient was determined by 2-stage PCR using universal and type-specific primers from the putative core region of the HCV genome by a modification of the method of Okamoto, et al.[22] and our previous report.[5] The genotype nomenclature was based on the system proposed by Simmonds, et al.[23]

Serum HCV RNA levels were determined by the second-generation Cobas Amplicor HCV Monitor assay (COBAS v2.0, Roche Diagnostics Systems, Meylan, France) (Amplicor monitor). The range of the linear relationship provided was 0.5×10^3 international unit per milliliter (kIU/mL) to 850 kIU/mL for Amplicor monitor.[24] Samples over 850 kIU/mL by Amplicor monitor were re-measured after 10 or 100 times dilution to determine accurate HCV RNA levels.

High-performance liquid chromatography

Seventeen generic SNMC drugs are approved for clinical use with patients in Japan. We analyzed the concentration of glycyrrhizin in SNMC and five of the generic drugs (Neophagen® and the other generic drug A (Glyphagen-C®), generic drug B (Hishipagen-C®), generic drug C (Kyominotin®), and generic drug D (Kebera-S®)) by a validated high-performance liquid chromatographic (HPLC) method. Neophagen and the generic drugs A to D were the top five sold in the Japanese market at the time of the study. The concentration of glycyrrhizin was determined by HPLC from 3 different lots of each drug. Five mL methanol was added to 10 μL aliquots of 20-times diluted saline from each drug. After mixing and centrifugation, the supernatant was decanted into another test tube with flushing nitrogen and evaporated at 50°C. After vortexing and centrifugation, 10 μL of the supernatant was injected into the HPLC system. The extract was separated on a Shimadzu-ODS (M) (Shimadzu, Kyoto, Japan) column with an acetonitrile/citrate buffer at a flow of 0.8 mL/min at the ambient temperature. Detection was ultraviolet absorption at 254 nm with the diode array detector by Shimadzu-C-R4A (Shimadzu).

Statistical Analysis

Continuous data were expressed as mean values± standard deviation (SD) of the mean. Statistical differences in the continuous data were determined by paired t-test, unpaired t-test, Kruskal-Wallis test, or Wilcoxon signed rank test, and categorical data were compared by chi-square test and Fisher's exact test.

A P value less than 0.05 was regarded as being statistically significant.

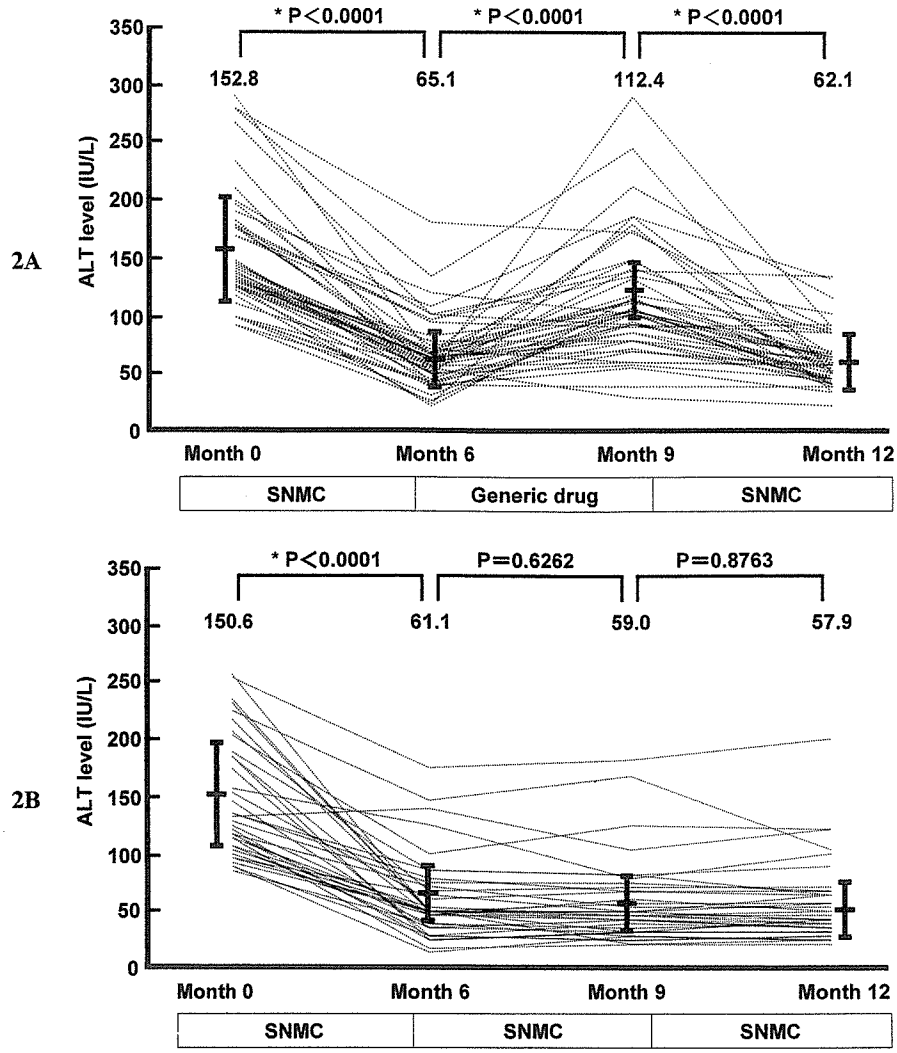
Conflict of Interest

We have no financial interests linked to this study.

RESULTS

Biochemical response to the initial SNMC treatment

After initial treatment with SNMC from Month 0 to Month 6, both Group A and Group B patients showed a significant decrease in mean ALT levels : Group A from 152.8±50.1 IU/L to 65.1±22.7 IU/L, and Group B from 150.6±49.2 IU/L to 61.1±30.7 IU/L (both $P < 0.0001$) (**Figures 2A and 2B**). The number of patients in both groups with an ALT level within 2 times ULN by SNMC, which indicated good response to SNMC, significantly increased during the period : Group A from 0% (0 of 46) to 73.9% (34 of 46), and Group B from 0% (0 of 46) to 75.0% (27 of 36) (both $P < 0.0001$). No significant difference in good response to SNMC was found between Groups A and B. Also, no



Figures 2A and 2B. Changes in serum alanine aminotransferase (ALT) levels in Groups A and B during the observation period. Figure 2A : Group A patients. Figure 2B : Group B patients. SNMC, Stronger Neo-Minophagen C.

significant difference between histological findings and response to SNMC was found either in Groups A or B.

Comparison of biochemical response of the switching and continuous treatment groups

From Month 6 to Month 9, Group A showed a significant increase in mean ALT levels (from 65.1±22.7 IU/L to 112.4±39.9 IU/L) ($P<0.0001$), whereas Group B showed no significant change (from 61.1±30.7 IU/L to 59.9±29.9 IU/L) ($P=0.6262$) (**Figures 2A and 2B**).

Figure 3 shows the rate of times increase of the serum ALT level of each patient from Month 6 to Month 9. In 46 Group A patients the following increases in ALT were observed : less than 1.25 times in 11 (23.9%) ; 1.25 times to 1.4 times in 9 (19.6%) ; 1.5 times to 1.9 times in 12 (26.1%) ; 2.0 times to less than 2.9 times in 7 (15.2%) ; and, 3.0 times or over in 7 (15.2%). For 36 Group B patients, the results were less than 1.25 times in 31 (86.1%) ; 1.25 times to less than 1.4 times in 4 (11.1%) ; 1.5 times to less than 1.9 times in 1 (2.8%) ; 2.0 times to less than 2.9 times in none ; and, 3.0 times or over in none.

A significant increase in mean ALT level was observed for the 34 Group A patients who had good response to the initial 6 months of SNMC treatment (from 41.9±9.9 IU/L to 110.0±48.8 IU/L) ($P<0.0001$). However, no significant increase was observed for the remaining 12 Group A patients without a good response (102.7±40.9 IU/L and 124.3±51.3 IU/L) ($P=0.0921$).

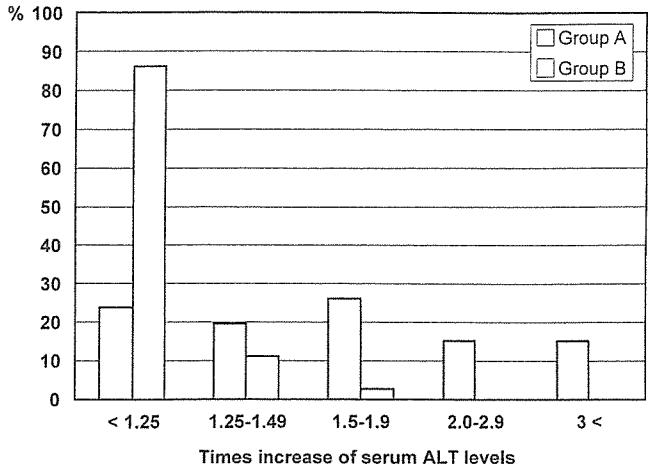


Figure 3. The rate of times increase of serum alanine aminotransferase (ALT) levels in Groups A and B from Month 6 to Month 9.

Table 2. Concentration of glycyrrhizin in SNMC and the five generic drugs, by the HPLC method

	Glycyrrhizin concentration	
	mg/ml**	Ratio to SNMC (%)
SNMC	2.00	—
Neophagen*	1.71	85.9
Generic drug A	1.88	94.4
Generic drug B	1.58	79.1
Generic drug C	1.65	82.7
Generic drug D	1.64	82.3

SNMC, stronger neo-minophagen C ; HPLC, high-performance liquid chromatographic

*was used in Group A patients of the present controlled study.

**shows the mean values from 3 different lots of each drug

The Group A patients returning to treatment with SNMC after switching to the generic drug showed a significant decrease in mean ALT level from Month 9 to Month 12 (from 112.4±39.9 IU/L to 62.1±23.0 IU/L) ($P<0.0001$) (**Figure 2A**). Continuous SNMC treatment of Group B patients resulted in no significant change of the mean ALT level during the same period (from 59.9±29.9 IU/L to 57.9±32.2 IU/L) ($P=0.8763$) (**Figure 2B**).

The above analysis was done in patients classified by histological findings and no significant difference between histological findings and treatment response was found either in Groups A or B.

Comparison of Virological response to switching and continuous treatment

The mean HCV RNA levels at Months 0, 6, and 9 were 953±246 kIU/mL, 1163±222 kIU/mL, and 1052±210 kIU/mL in Group A, and 1156±289 kIU/mL, 1313±249 kIU/mL, and 1284±328 kIU/mL in Group B. No significant differences in serum mean HCV RNA levels during the period were observed in either Group A or B patients. No patients had viremic clearance during the treatment.

Comparison of adverse reactions to switching and continuous treatment

The expected adverse effects of glycyrrhizin preparation administration include pseudo-aldosteronism with hypokalemia, sodium retention, elevated blood pressure, and retention of body fluids. No Group A or B patients had any symptoms of pseudo-aldosteronism. The mean serum potassium levels at Months 0, 6, and 9 did not significantly decrease in Group A or B patients : Group A, 4.3±0.4 mMol/L, 4.1±0.4 mMol/L and 4.2±0.3 mMol/L ; Group B, 4.0±0.4 mMol/L, 4.0±0.3 mMol/L, and 4.2±0.3 mMol/L. The mean systolic blood pressure at Months 0, 6, and 9 did not significantly rise in Group A or B patients : Group A, 129.1±19.9 mmHg, 130.3±23.3 mmHg, 132.1±18.7 mmHg ; Group B, 131.1±15.7 mmHg, 134.1±19.2 mmHg, and 132.1±13.9 mmHg.

Concentration of glycyrrhizin in SNMC and the five other generic drugs by HPLC

Table 2 shows the average concentration of glycyrrhizin in 3 different lot numbers of each drug, determined by HPLC. The average concentration of glycyrrhizin in Neophagen and generic drugs A to D was lower than that in SNMC, with a range in the ratio to SNMC of from 79.1 to 94.4%. No significant difference was found in the concentration of glycyrrhizin among different lot numbers of each drug.

DISCUSSION

To our knowledge, no study comparing the clinical efficacy of SNMC, the original preparation of glycyrrhizin, with any other generic drugs has been published. Although the study was not randomized or blinded, we were able to document differences in efficacy between SNMC and the generic drugs in a longitudinally controlled study of chronic hepatitis C patients.

The effect on ALT of the 6-month SNMC treatment was significantly more often lost in the patients that switched to the generic drug than in those receiving continuous treatment, especially among patients with an initially good response. These findings suggest that a blind, randomized, control trial of the effectiveness of SNMC and its generic drugs is needed.

SNMC, the most popular glycyrrhizin based drug in Japan, is derived from the roots of the plant *Glycyrrhiza glabra* (licorice). Glycyrrhizin has been used for the treatment of chronic hepatitis for more than 20 years in Japan.[13] The exact mechanism by which glycyrrhizin reduces the progression of liver disease without clearing the virus is unknown. A few in vitro and animal studies suggest that glycyrrhizin or its metabolite glycyrrhetinic acid inhibits lipid peroxidation, thereby protecting the hepatocytes.[22]

It was reported that up to half of patients with chronic HCV infection treated with SNMC either had an improved or normal ALT level, depending on the frequency of SNMC administration.[23] The effect on ALT was also lost after cessation of SNMC treatment, suggesting a rebound phenomenon. The present study found SNMC to have a 5.6% to 20.9% higher concentration of glycyrrhizin than the generic drugs, even though the package inserts stated that each drug contained 2 mg of glycyrrhizin per mL in saline. The Japanese Pharmacopoeia states that any glycyrrhizin drug must be a derivative of licorice and contain at least 2.5% glycyrrhizin. Glycyrrhizin based drugs cannot be synthesized, but must be a naturally occurring compound. Therefore, the drug purity depends on the purification process : the method of licorice extraction, the temperature, time, solvent, and medium used for purification, and the purifying method, HPCL or absorption. It is possible that the processes used resulted in the observed differences in glycyrrhizin concentration in the SNMC and the generic drugs tested in this study. Whatever the reason for these differences, however, our findings suggest that these differences in concentration lead to differences in efficacy in reducing the ALT level of the patients switching to generic drug.

The efficacy of glycyrrhizin for liver disease has been well documented in Japan. Although SNMC decreases the ALT level in chronic hepatitis C patients, SNMC does not have any significant effect on viral clearance.[24] We also documented no significant effect on the HCV RNA level by either SNMC or the generic drugs. However, SNMC is used extensively for chronic hepatitis C, in particular for patients who did not respond to IFN treatment. SNMC has been reported to reduce the cumulative risk of HCC by more than half, from 25% to 12% at 15 years.[15] Glycyrrhizin acts in a cytoprotective manner, possibly by its ability to inhibit tumor necrosis factor α-mediated apoptosis[25] and/or by inhibition of anti-Fas antibody-induced hepatitis.[26]

It is also very important to consider cost-effectiveness analysis of the generic drugs. The utilization of low price generic drugs has been promoted to minimize the cost of drugs in Japan. The difference in price of a single ampoule of SNMC and the generic drugs is under one U. S. dollar. However, because nearly 100 million ampoules of SNMC are used each year in Japan for the treatment of chronic hepatitis, even a slight difference in price of one ampoule leads to a significant difference in total drug cost. Nevertheless, the lower clinical efficacy of the generic drugs does not warrant their use, even though there may be a substantial cost savings.

Although we already reported a part of the present study in a Japanese journal,[27] the present study includes additional data about the influence on HCV RNA levels by intravenous glycyrrhizin and the concentrations of glycyrrhizin among different glycyrrhizin products.

In concluding, one potential limitation of the present study should be noted : There may be study bias, because we divided our patients into two groups, SNMC and generic drug treatment groups, according to treatment hospitals. Nevertheless, the two groups of patients had no significant difference in baseline data. Therefore, we believe that our findings offer physicians important information on the efficacy of SNMC and similarly used generic drugs. In conclusion, SNMC and the generic drugs differed in their ability to reduce ALT in patients with chronic hepatitis C.

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THE EMBO JOURNAL

Hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90

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Hepatitis C virus (HCV) nonstructural protein 5A (NS5A) is a component of viral replicase and is well known to modulate the functions of several host proteins. Here, we show that NS5A specifically interacts with FKBP8, a member of the FK506-binding protein family, but not with other homologous immunophilins. Three sets of tetratricopeptide repeats in FKBP8 are responsible for interactions with NS5A. The siRNA-mediated knockdown of FKBP8 in a human hepatoma cell line harboring an HCV RNA replicon suppressed HCV RNA replication, and this reduction was reversed by the expression of an siRNA-resistant FKBP8 mutant. Furthermore, immunoprecipitation analyses revealed that FKBP8 forms a complex with Hsp90 and NS5A. Treatment of HCV replicon cells with geldanamycin, an inhibitor of Hsp90, suppressed RNA replication in a dose-dependent manner. These results suggest that the complex consisting of NS5A, FKBP8, and Hsp90 plays an important role in HCV RNA replication.

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Introduction

Hepatitis C virus (HCV) persistently infects approximately 170 million people worldwide, and it is responsible for most cases of severe chronic liver diseases, including cirrhosis and hepatocellular carcinoma (Wasley and Alter, 2000). Although treatment with interferon (IFN) alpha and ribavirin is available for about half of the population of HCV patients (Manns *et al*, 2001), therapeutic and preventative vaccines are still necessary for more effective treatment; however, such vaccines have not yet been developed. HCV belongs to the *Flaviviridae* family and possesses a positive-sense single-stranded RNA with a nucleotide length of 9.6 kb. The HCV genome encodes a single large precursor polyprotein composed of about 3000 amino acids, and the polyprotein is processed by cellular and viral proteases into at least 10 structural and nonstructural (NS) proteins (Moriishi and Matsuura, 2003).

The development of efficient therapies for hepatitis C has been hampered by the lack of a reliable cell-culture system, as well as by the absence of a non-primate animal model. The HCV replicon consists of an antibiotic selection marker and a genotype 1b HCV RNA, which replicates autonomously in the intracellular compartments in a human hepatoma cell line, Huh7 (Lohmann *et al*, 1999). This replicon system has functioned as an important tool in the investigation of HCV replication and it has served as a cell-based assay system for the evaluation of antiviral compounds. Recently, cell culture systems for *in vitro* replication and infectious viral production were established based on the full-length HCV genome of genotype 2a, which was isolated from an HCV-infected patient who developed fulminant hepatitis (Lindenbach *et al*, 2005; Wakita *et al*, 2005; Zhong *et al*, 2005). However, no robust *in vitro* culture systems for the 1a and 1b genotypes, which are the most prevalent HCV genotypes in the world, have been established to date.

Several viruses require viral and host molecular chaperones for entry, replication, and assembly, as well as for other steps in viral production (Maggioni and Braakman, 2005; Mayer, 2005). Cyclosporine A has been found to effectively inhibit viral replication in hepatitis C patients and in HCV replicon cells (Inoue *et al*, 2003; Watashi *et al*, 2003). Recently, it was shown that cyclophilin (Cyp) B specifically binds to NS5B and promotes association with the genomic RNA; furthermore, cyclosporine A was shown to disrupt interactions between NS5B and CypB (Watashi *et al*, 2005). CypB belongs to the immunophilin family, which shares peptidyl propyl *cis/trans* isomerase (PPIase) activity and an affinity for the immunosuppressive drug (Fischer and Aumuller, 2003). Furthermore, blockades of CypA, CypB, and CypC, as well as the induction of cellular stress responses, have been suggested to be involved in cyclosporine A-induced reduction of HCV RNA replication (Nakagawa *et al*, 2005). However, the involvement of other immunophilins in HCV RNA replication is not yet well understood.

HCV nonstructural protein 5A (NS5A) is a membrane-anchored phosphoprotein that possesses multiple functions in viral replication, IFN resistance, and pathogenesis (Macdonald and Harris, 2004). NS5A contains a zinc metal-binding motif within the N-terminal domain, and this zinc-binding ability is known to be essential for HCV replication (Tellinghuisen *et al*, 2004, 2005). Adaptive mutations frequently mapped in the coding region of NS5A have been shown to increase RNA replication (Yi and Lemon, 2004; Appel *et al*, 2005) and they are known to affect the hyperphosphorylation of NS5A by an unknown host kinase (Koch and Bartenschlager, 1999; Neddermann *et al*, 1999; Pietschmann

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et al, 2001). RNA replication in HCV replicon cells has been shown to be inhibited by treatment with lovastatin, a drug that decreases the production of mevalonate by inhibiting 3-hydroxy-3-methylglutaryl CoA reductase; this inhibition of RNA replication was reversed by the addition of geranylgeraniol, which suggests that HCV RNA replication requires geranylgeranylated proteins (Ye *et al*, 2003; Kapadia and Chisari, 2005). A NS5A-pull-down assay identified a geranylgeranylated protein, FBL2, as a NS5A-binding protein (Wang *et al*, 2005). Although several host proteins could potentially interact with NS5A, little is known about NS5A function.

To gain a better understanding of the functional role of NS5A in HCV replication, we screened human libraries by employing a yeast two-hybrid system and using NS5A as bait. We thereby successfully identified FKBP8 as an NS5A-binding protein. FKBP8 is classified as a member of the FK506-binding protein family, but it lacks several amino-acid residues thought to be important for PPIase activity and FK506 binding (Lam *et al*, 1995). We demonstrated here that FKBP8 forms a complex with Hsp90 and NS5A, and that this complex is critical for HCV replication, as based on the finding that treatment of the HCV replicon cells with geldanamycin, an inhibitor of Hsp90, suppressed RNA replication. These results therefore suggest that protein complex formation with NS5A, FKBP8, and Hsp90 plays a crucial role in HCV RNA replication.

Results

Identification of human FKBP8 as an HCV NS5A-binding partner

To identify host proteins that specifically interact with NS5A, we screened human brain and liver libraries using a yeast two-hybrid system that employs NS5A as bait. One positive clone was isolated from among 2 million colonies of the human fetal brain library, and the nucleotide sequence of this clone was determined. Several positive clones were isolated from the human liver library, but most of these clones included exon fragments of other than FKBP and/or noncoding regions. A BLAST search revealed that the positive clone encodes a full-length coding region of FKBP38, human FK506-binding protein 38 kDa. Although FKBP38 has been isolated from human and mouse mRNA (Lam *et al*, 1995), an additional sequence at the N-terminus of FKBP38 was revealed based on an analysis of the transcriptional start site in the genomic sequences of FKBP38 (Nielsen *et al*, 2004). The isoforms of FKBP38 were designated as FKBP8, which includes splicing variants of 44 and 46 kDa in mice, and 45 kDa in humans corresponds to the 44 kDa of the mouse FKBP8 (Nielsen *et al*, 2004). Human FKBP8 is identical to FKBP38 except for the extra 58 amino-acid residues at the N-terminus, and the FK506-binding domain in the N-terminal half, followed by three sets of tetratricopeptide repeats (TPRs), a calmodulin binding site, and a transmembrane domain (Figure 1A). Because the levels of expression of FKBP8 and FKBP38 have not been well characterized in human cell lines, we generated a mouse monoclonal antibody against human FKBP8, and we designated it as clone KDM19. This antibody recognizes a 50-kDa of endogenous FKBP8 in 293T cells, as well as exogenous HA-tagged FKBP8 (HA-FKBP8), which has slightly greater molecular weight (Figure 1B). Although the KDM19 antibody detected an exogenous HA-tagged FKBP38 (HA-FKBP38) in 293T cells, no protein band corresponding to

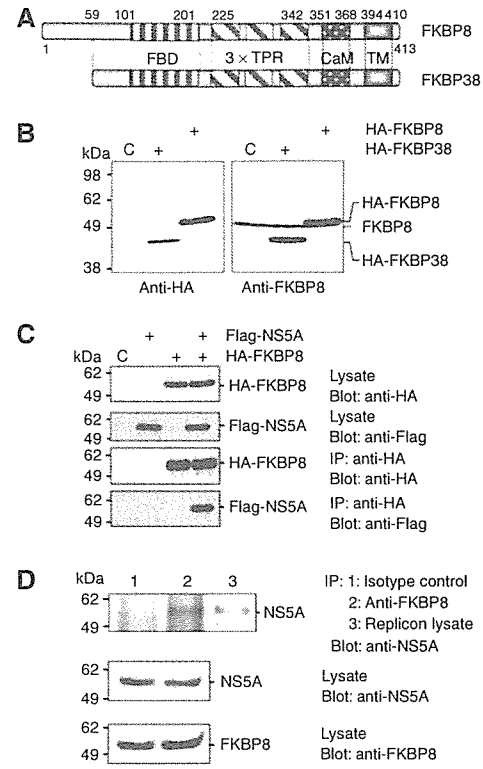

Figure 1 Expression of FKBP8 and FKBP38 in mammalian cells. (**A**) Schematic representation of FKBP8 and FKBP38. The FK506-binding domain (FBD), tetratricopeptide repeat (TPR), putative calmodulin binding motif (CaM), and transmembrane domain (TM) are shown. (**B**) N-terminally HA-tagged FKBP8 and FKBP38 were expressed in 293T cells and visualized by immunoblotting using mouse monoclonal antibody to FKBP8 or the HA tag. (**C**) HA-FKBP8 was expressed together with Flag-NS5A of genotype 1b (J1) in 293T cells and was immunoprecipitated with anti-HA antibody. Immunoprecipitated proteins were subjected to immunoblot with anti-Flag or HA antibody. (**D**) Endogenous FKBP8 in HCV replicon (9–13) cells was immunoprecipitated with isotype control (lane 1) or anti-FKBP8 antibody, KDM-11 (lane 2). Endogenous FKBP8 was co-immunoprecipitated with HCV NS5A. The data shown in each panel are representative of three independent experiments.

endogenous FKBP38 was detected. Similar results were obtained in human liver tissue and in the hepatoma cell lines Huh7, HepG2, and FLC-4 (data not shown). These findings suggest that FKBP8, but not FKBP38, is a major product in human cells. In order to examine whether or not FKBP8 binds to NS5A protein in mammalian cells, Flag-tagged NS5A (Flag-NS5A) was expressed together with HA-FKBP8 in 293T cells. Cells transfected with the expression plasmids were harvested at 48-h post-transfection, lysed, and subjected to immunoprecipitation. Flag-NS5A was co-precipitated with HA-FKBP8 by anti-HA antibody (Figure 1C). Flag-NS5A was also immunoprecipitated together with HA-FKBP38, suggesting that the extra N-terminal sequence of FKBP8 is not critical for NS5A binding (data not shown). To further confirm the specific interaction of HCV NS5A with endogenous FKBP8, this interaction was examined in Huh7(9–13) cells harboring subgenomic HCV RNA replicon. Endogenous FKBP8 was co-precipitated with HCV NS5A by anti-FKBP8 antibody (Figure 1D). To determine the direct interaction between FKBP8 and NS5A, His_6-tagged FKBP8 (His-FKBP8) and thioredoxin-fused domain 1 of NS5A (Trx-NS5A) prepared in *Escherichia coli* were examined by pull-down

analysis. Trx-NS5A was co-precipitated with His-FKBP8 by anti-FKBP8 antibody (Supplementary Figure 1), suggesting that FKBP8 can directly bind to NS5A domain I.

In order to investigate the interaction of FKBP8 with the NS5A of other HCV genotypes, HA-tagged NS5A (HA-NS5A) proteins of genotype 1a (H77C), 1b (Con1 and J1), or 2a (JFH1) were expressed together with Flag-tagged FKBP8 (Flag-FKBP8) in 293T cells (Figure 2A). Flag-FKBP8 was co-immunoprecipitated with the HA-NS5As of all of the genotypes examined here by anti-HA antibody, although it should be noted that the interaction between Flag-FKBP8 and the HA-NS5A of genotype 2a was weaker than that of the other genotypes tested. Furthermore, the HA-NS5As were co-precipitated with Flag-FKBP8 by anti-Flag antibody (Figure 2A, bottom panel). The TPR domain of FKBP8 is known to be responsible for protein–protein interactions. Among the immunophilins, FKBP8 shares high homology with CypD and FKBP52, both of which contain three tandem repeats of TPR, as does FKBP8 (Boguski *et al*, 1990; Hirano *et al*, 1990). However, co-immunoprecipitation of Flag-NS5A with HA-FKBP52 and HA-CypD by anti-Flag or anti-HA antibody was not successful (Figure 2B). These results indicate that FKBP8 specifically interacts with NS5A.

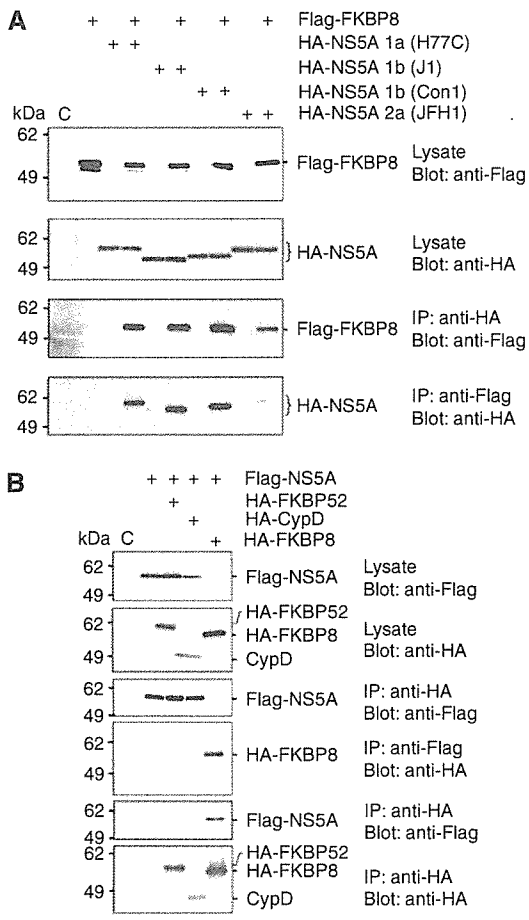

Figure 2 Specific interaction between FKBP8 and NS5A. (**A**) HA-NS5As were obtained from several genotypes of HCV and were expressed with Flag-FKBP8 in 293T cells. Proteins immunoprecipitated with anti-HA or Flag antibody were subjected to Western blotting. (**B**) Flag-NS5A was coexpressed with HA-FKBP8, -CypD, or -FKBP52 in 293T cells. Proteins immunoprecipitated with anti-HA or -Flag tag antibody were subjected to Western blotting. The data shown in each panel are representative of three independent experiments.

The TPR domain is required for the interaction between NS5A and FKBP8

FKBP8, CypD, and FKBP52 have high similarity and identity to each other within the TPR domain (Lam *et al*, 1995). Several FKBP8 mutants lacking the transmembrane region, the calmodulin-binding region, the TPR domains, and/or the FK506-binding domain were generated in order to identify the region responsible for the interaction with NS5A (Figure 3A). HA-tagged FKBP8 mutants were coexpressed with Flag-NS5A in 293T cells and were immunoprecipitated with anti-HA antibody. Flag-NS5A was co-immunoprecipitated with the FKBP8 mutants, except in the case of a dTPR mutant lacking the transmembrane, calmodulin binding, and TPR domains (Figure 3B). Although the level of expression of dFBD, an FKBP8 mutant with a deletion in the N-terminal region containing the FK506-binding domain, was lower than that of dTPR, co-immunoprecipitated NA5A was clearly detected. These findings suggested that the lack of an association of dTPR with NS5A was not due to the relatively low level of expression of dTPR, as compared to those of the other FKBP8 mutants. A specific interaction of NS5A with the TPR domain, but not with the transmembrane, calmodulin binding, or FK506-binding domains of FKBP8, was also observed using the yeast two-hybrid system (data not shown). These results indicated that FKBP8 interacts with HCV NS5A through the TPR domain.

FKBP8 forms a homomultimer and a heteromultimer with NS5A

FKBP8 is similar to FKBP52 and CypD with respect to their amino-acid sequences and functional domains. In order to examine the interactions among FKBP8, FKBP52, and CypD,

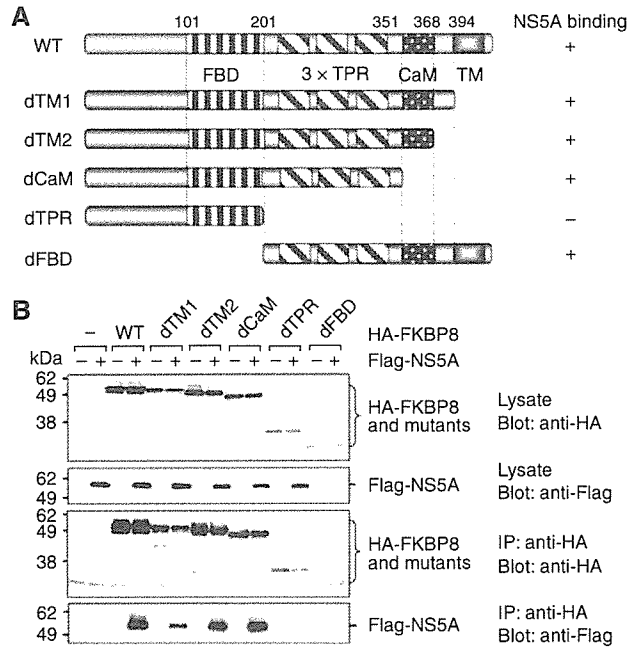

Figure 3 Determination of the NS5A-binding region in FKBP8. (**A**) Schematic representation of FKBP8 and deleted mutants. (**B**) Flag-NS5A was coexpressed with HA-FKBP8 and its mutants in 293T cells. Proteins immunoprecipitated with anti-HA antibody were subjected to Western blotting. The data shown in each panel are representative of three independent experiments.

Flag-FKBP8 was coexpressed with HA-FKBP52, HA-CypD, or HA-FKBP8 in 293T cells and it was immunoprecipitated with anti-Flag or anti-HA antibody. Flag-FKBP8 and HA-FKBP8 were co-immunoprecipitated with each antibody, but not with HA-FKBP52 or HA-CypD. It is known that Hsp90 forms a homodimer and also interacts with FKBP52 through TPR domain as FKBP8 (Chadli *et al*, 2000). If homodimer of FKBP8 is due to intermediating of Hsp90 as FKBP8-Hsp90-Hsp90-FKBP8 complex, FKBP52 would be co-precipitated with FKBP8 as FKBP8-Hsp90-Hsp90-FKBP52. However, we could not detect any association of FKBP8 and FKBP52 in the immunoprecipitation analysis (Figure 4A). These data suggest that FKBP8 can form a homomultimer without Hsp90 and associate with neither FKBP52 nor CypD through Hsp90. To examine the effects of the interaction with NS5A on the homomultimerization of FKBP8, HA-NS5A was co-expressed with Flag-FKBP8 and Glu-Glu-tagged FKBP8 (EE-FKBP8) in 293T cells, and was then immunoprecipitated with anti-Flag or anti-EE antibody. HA-NS5A was co-immunoprecipitated with Flag-FKBP8 and EE-FKBP8 by anti-Flag or anti-EE antibody (Figure 4B). Although multimerization of EE-FKBP8 and Flag-FKBP8 was increased about 2 times in the presence of HA-NS5A, but no further increase of the multimerization of FKBP8 was observed by the increase of HA-NS5A expression (Figure 4C). These results further support the notion that NS5A binds to FKBP8 via the TPR domain and slightly influence homomultimerization exerted by the FK506-binding domain.

Knockdown of FKBP8 decreases RNA replication in HCV replicon cells

In order to determine the role of endogenous FKBP8 on HCV RNA replication, 80 nM of small interfering RNA (siRNA) targeted to FKBP8 or control siRNA was transfected into Huh7 (9–13) cells harboring subgenomic HCV replicon RNA. To verify the specificity of the knockdown of FKBP mRNA, we synthesized three siRNAs targeted to different regions of FKBP8 (Targets 1–3). The total RNA was extracted from the transfected cells, and HCV RNA and FKBP8 mRNA levels were determined by real-time polymerase chain reaction (PCR). HCV subgenomic RNA and FKBP8 mRNA levels in the cells transfected with each of the FKBP8 siRNAs were reduced by more than 60%, as compared to the levels in cells treated with the control siRNA at 72 h post-transfection (Figure 5A). The levels of expression of FKBP8 and the HCV proteins (i.e., NS4B, NS5A, and NS5B) decreased in HCV replicon cells transfected with 80 or 160 nM of the FKBP8 siRNA (Target 1), but this was not observed in the cells with the control siRNA (Figure 5B). To further confirm the specificity of the reduction in HCV RNA replication in the replicon cells putatively achieved by the knockdown of FKBP8, a plasmid encoding Flag-FKBP8 containing either a silent mutation within the siRNA target sequence (Flag-rFKBP8) or empty plasmid was transfected into the HCV replicon cells and then selection was carried out with the appropriate antibiotics. The remaining cells, that is, Huh7rFKBP8 and Huh7c cells, harboring the Flag-rFKBP8

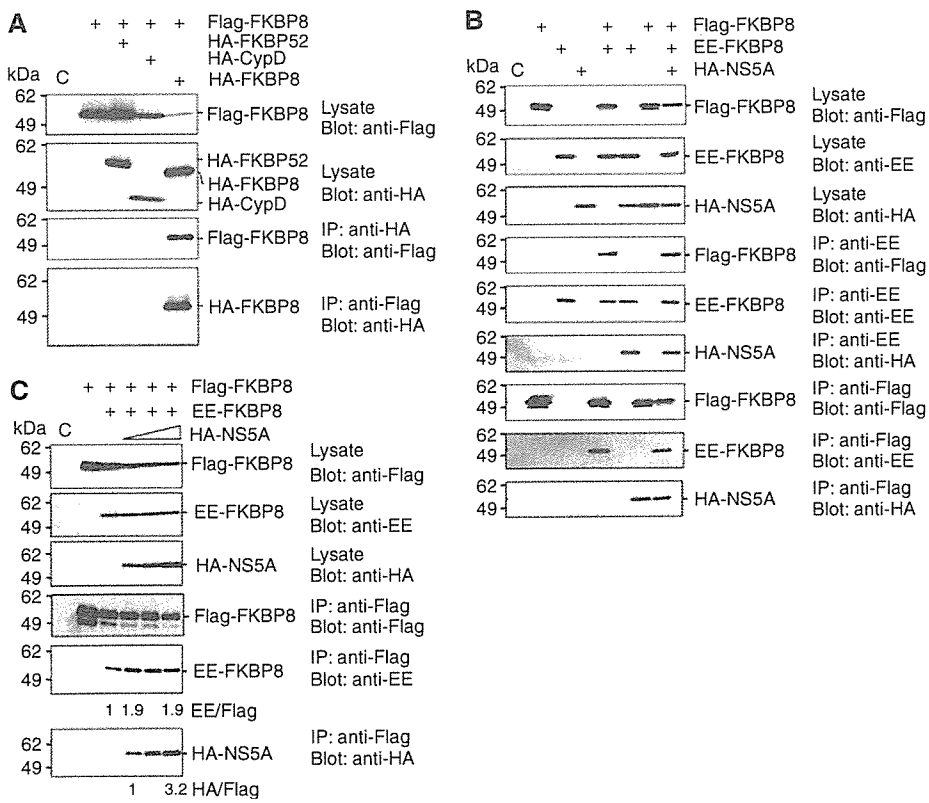

Figure 4 Homomultimerization of FKBP8. (**A**) Flag-FKBP8 was coexpressed with HA-FKBP52, -CypD, or -FKBP8 in 293T cells, and was immunoprecipitated with anti-HA or Flag antibody. Precipitates were analyzed by Western blotting. (**B**) Flag- or EE-tagged FKBP8 was coexpressed with HA-NS5A in 293T cells and was immunoprecipitated with anti-EE or Flag antibody. Precipitates were analyzed by Western blotting. (**C**) Flag- and EE-tagged FKBP8 were coexpressed with increasing amounts of HA-NS5A (0.1, 0.2, and 0.4 μg of expression plasmid/well) in 293T cells. Immunoprecipitates with anti-Flag antibody were analyzed by Western blotting. The data shown in each panel are representative of three independent experiments.

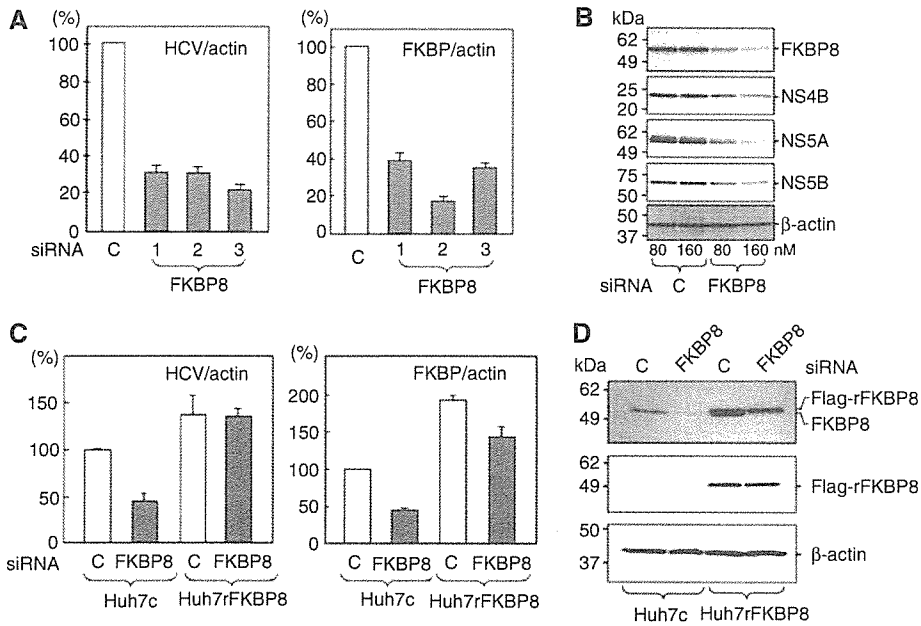

Figure 5 Decrease in HCV RNA by FKBP8-targeted siRNA. (**A**) HCV replicon cells (9–13 cells) were transfected with each of three kinds of siRNA targeted to FKBP8 or nontargeted siRNA at a final concentration of 80 nM. Transfected cells were collected at 72 h post-transfection, and FKBP8 mRNA and HCV RNA levels were determined by real-time PCR after being normalized with β-actin mRNA. (**B**) HCV replicon cells transfected with 80 and 160 nM of Target 1 or nontargeted siRNA were harvested at 72 h post-transfection, and the samples were analyzed by immunoblotting. (**C**) HCV replicon cells expressing Flag-rFKBP8 mutant (Huh7rFKBP8) or control cells (Huh7c) were transfected with Target 1 (gray bars) or nontargeted (white bars) siRNA at a concentration of 80 nM. Transfected cells were harvested at 72 h post-transfection, and HCV RNA (left) and FKBP8 mRNA (right) were measured by real-time PCR and expressed as % increase after being normalized with the expression of β-actin mRNA. (**D**) Levels of expression of endogenous FKBP8, exogenous Flag-rFKBP8, and β-actin in the replicon cells after transfection of the siRNAs were determined by immunoblotting using specific antibodies. The data shown in each panel are representative of three independent experiments.

and empty plasmid, respectively, were pooled and then transfected with the FKBP8 siRNA (Target 1) or control siRNA. Although transfection of the FKBP8 siRNA led to a 60% reduction of HCV RNA and FKBP8 mRNA in Huh7c cells, in comparison with levels in cells transfected with the control siRNA, no reduction in HCV RNA, and only a slight reduction in FKBP8 mRNA levels were observed in Huh7rFKBP8 cells (Figure 5C). Flag-rFKBP8 expression was clearly detected in Huh7rFKBP8 cells after transfection with the FKBP8 siRNA or control siRNA, whereas the endogenous FKBP8 decreased in both Huh7rFKBP8 and Huh7c cells with the FKBP8 siRNA (Figure 5D). These findings suggest that the slight reduction of FKBP8 mRNA in the Huh7rFKBP8 cells was due to a loss of endogenous FKBP8. Knockdown of FKBP8 by siRNA induce no apoptosis in a hepatoma cell line (Supplementary Figure 2). These results therefore confirmed that the inhibition of HCVRNA replication by FKBP8 siRNA was due to a specific reduction in the mRNA of FKBP8, but was not due to a nonspecific reduction of any other host mRNA.

To further examine the involvement of FKBP8 on HCV replication, we established a line of Huh7 cells that stably expresses shRNA targeted to FKBP8. Huh7 was transfected with pSilencer 2.1 U6 hygro containing the cDNA of shRNA to FKBP8, and then selection was carried out with hygromycin. FKBP8 was detected in Huh7 cells harboring a control plasmid (Huh7N), whereas decreased expression of FKBP8 was clearly observed in cells expressing the shRNA to FKBP8 (Huh7FKBP8KD) (Figure 6A). In order to examine the effects of the knockdown of FKBP8 on HCV RNA replication, a chimeric HCV RNA containing the *Renilla* luciferase gene was transfected into these cell lines. Although the chimeric HCV RNA exhibited 5.5 times higher replication than a replication deficient GND mutant RNA in Huh7N, only a doubling of the levels of replication was observed in Huh7FKBP8KD (Figure 6B). Furthermore, HCV RNA containing a neomycin-resistant gene was transfected into the cell lines in order to examine the role played by FKBP8 in HCV RNA replication. The efficiency of colony formation in Huh7N and Huh7FKBP8KD cells with the HCV RNA were 1700 and 23 colonies/μg RNA, respectively (Figure 6C). We also examined the role of FKBP8 on the cell culture system for HCV infection. The siRNA-mediated knockdown of FKBP8 impaired both intracellular viral RNA replication and release of HCV core protein into the culture supernatants (Figure 6D). These results further confirmed that FKBP8 plays a crucial role in the efficient replication of HCV RNA.

FKBP8 forms a multicomplex with NS5A and Hsp90

To identify the cellular proteins that associate with FKBP8, we employed a purification strategy using an MEF affinity tag composed of myc and FLAG tags fused in tandem and separated by a spacer sequence containing a TEV protease cleavage site (myc-TEV-FLAG) (Ichimura *et al*, 2005). The MEF expression cassette fused with FKBP8 was transfected into 293T cells and the cells were immunoprecipitated. The endogenous FKBP8-binding proteins bound to the Flag beads were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and were then visualized by silver staining. The visible protein bands were excised and determined by a nanoflow LC-MS/MS system. Major protein bands with a molecular size of 94 and 53 kDa were identified as Hsp90 and FKBP8, respectively, although it should be

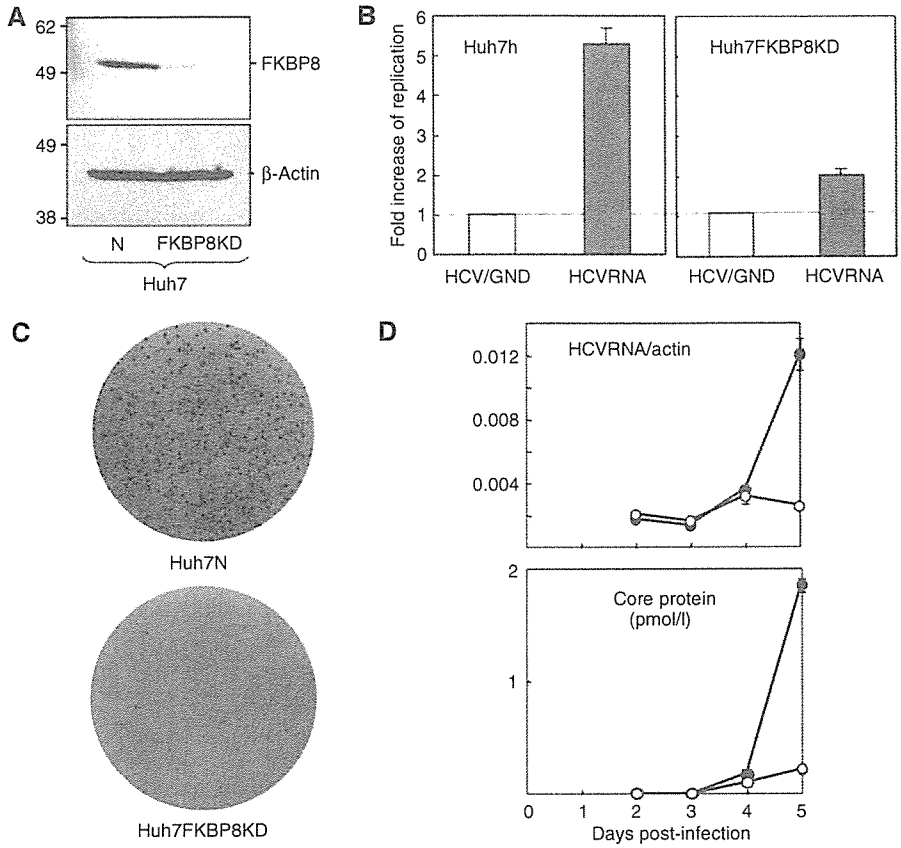

Figure 6 Effect of knockdown of FKBP8 on the transient replication, colony formation, and viral infection. (**A**) Levels of expression of FKBP8 and β-actin in Huh7N and Huh7 FKBP8KD cell lines bearing plasmids encoding shRNA for control mRNA (lane 1) and for FKBP8 mRNA (lane 2), respectively. (**B**) Each cell line was transfected with *in vitro*-transcribed HCV replicon RNA, pFK-I_{389} hRL/NS3-3′/NK5.1 (HCVRNA), or a replication-negative mutant, pFK-I_{389} hRL/NS3-3′/NK5.1GND (HCV/GND). The fold increase in replication was determined by the increase in luciferase activity at 48 h compared with that observed 4 h after standardization, as based on the activity of the replication-deficient HCV/GND replicon. (**C**) Huh7N and Huh7 FKBP8KD cell lines were transfected with *in vitro*-transcribed replicon RNA (pFK-I_{389} neo/NS3-3′/NK5.1) and the cells were incubated for 4 weeks. The remaining cells were fixed with 4% paraformaldehyde and then were stained. (**D**) Huh7.5.1 cells were transfected with either of siRNA targeted to FKBP8 (Target 1) or nontarget control at a concentration of 80 nM. The cells were inoculated with HCVcc at 24 h after transfection and cells and culture supernatants were harvested every day. Intracellular viral RNA (upper) and HCV core protein in the supernatant (lower) were determined. The data shown in each panel are representative of three independent experiments.

noted that the remaining bands detected in the samples could not be reliably identified (Figure 7A).

In order to elucidate the interaction of Hsp90 with FKBP8 in mammalian cells, Flag-FKBP8 was coexpressed with HA-Hsp90 and immunoprecipitated by anti-Flag or anti-HA antibody. HA-Hsp90 and Flag-FKBP8 were co-precipitated with each other by either of the antibodies but no interaction was observed between HA-Hsp90 and Flag-NS5A (Figure 7B). To examine the interplay among NS5A, FKBP8, and Hsp90, HA-Hsp90 was coexpressed with EE-FKBP8 and/or Flag-NS5A (Figure 7C). Co-immunoprecipitation of Hsp90 and NS5A was clearly detected in the presence but not in the absence of FKBP8. The increase in NS5A expression had no effect on the interaction between FKBP8 and Hsp90 (Supplementary Figure 3). These results suggest that Hsp90 does not directly bind to NS5A but forms complex with NS5A through the interaction with FKBP8.

FKBP8 interacts with NS5A and Hsp90 via different sites in the TPR domain

Crystal structure of the TPR domain of Hop, an adaptor chaperone that binds both Hsp70 and Hsp90, revealed that C-terminal MEEVD motif of Hsp90 is held by amino-acid residues of the two-carboxylate clamp positions within the TPR domain (Scheufler *et al*, 2000; Brinker *et al*, 2002; Cliff *et al*, 2006). To examine the role of the C-terminal MEEVD motif of Hsp90 on the interaction with FKBP8, Hsp90 mutant lacking the MEEVD motif (HA-Hsp90ΔMEEVD) was co-expressed with Flag-FKBP8 (Figure 8A). Wild-type Hsp90 but not the mutant Hsp90 was co-precipitated with FKBP8, indicating that the FKBP8 interacts with Hsp90 via the C-terminal MEEVD motif. Lys^{307} and Arg^{311} residues in the two-carboxylate clamp positions of FKBP8 were conserved among the TPR domain of other immunophilins, such as FKBP52 and CypD (Figure 8B). To examine the role of the two-carboxylate clamp positions of FKBP8 for the interaction with Hsp90 and NS5A, FKBP8 mutant replaced Lys^{307} and Arg^{311} with Ala, designated as FKBP8TPRmut, was coexpressed with HA-Hsp90 or HA-NS5A (Figure 8C). FKBP8TPRmut exhibited no interaction with Hsp90, but still retained the capability of binding to NS5A, indicating that FKBP8 interacts with Hsp90 and NS5A through the conserved two-carboxylate clamp residues and other region in the TPR domain, respectively.

Hsp90 participates in the replication of HCV RNA

To examine the role of Hsp90 in the replication of HCV RNA, FKBP8TPRmut lacking the ability to bind to Hsp90 was

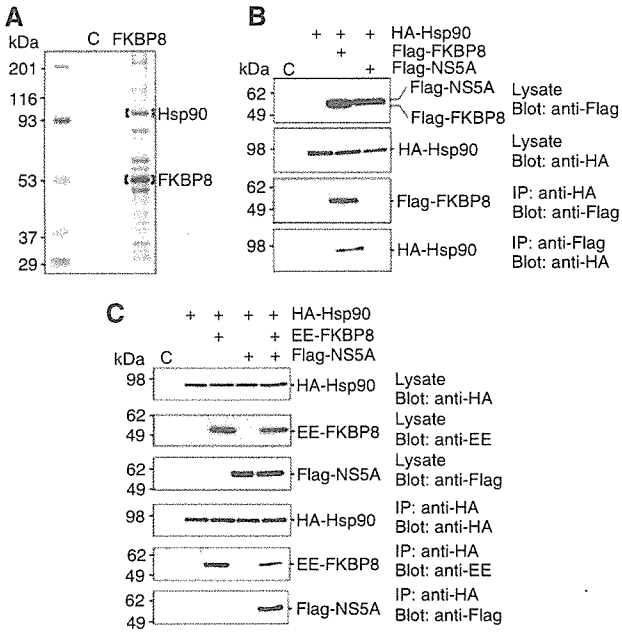

Figure 7 FKBP8 forms complex with NS5A and Hsp90. (**A**) An N-terminally myc-TEV-Flag-tagged FKBP8 was expressed in 293T cells and immunoprecipitated. The precipitated proteins were applied to SDS–PAGE and then stained with silver staining. Hsp90 and FKBP8 were identified by LC-MS/MS. (**B**) HA-Hsp90 was coexpressed with Flag-FKBP8 or Flag-NS5A in 293T cells, and was immunoprecipitated by anti-HA or anti-Flag antibody. Precipitates were analyzed by Western blotting. (**C**) HA-Hsp90 was coexpressed with EE-FKBP8 and/or Flag-NS5A in 293T cells and was immunoprecipitated with anti-HA antibody. Precipitates were analyzed by Western blotting by anti-EE, -HA or -Flag antibody.

expressed in HCV replicon cells (Figure 8D). Expression of FKBP8TPRmut resulted in 30% reduction of HCV RNA replication, suggesting that FKBP8TPRmut works as a dominant negative. Geldanamycin is well known to bind to the ATP/ADP binding site of Hsp90 and specifically inhibits the enzymatic activity of Hsp90, resulting in the promotion of the degradation of client proteins for Hsp90 (Neckers, 2002). To determine the effects of Hsp90 inhibition induced by geldanamycin on the replication of HCV RNA, HCV replicon cells were treated with various concentrations of geldanamycin. Treatment with geldanamycin clearly reduced the levels of HCV RNA replication (Figure 8E); moreover, this treatment led to the slight suppression of NS5A without reducing the levels of FKBP8 expressed in the HCV replicon cells (Figure 8F). Although the inhibition of cleavage at the NS2/NS3 junction by geldanamycin has been demonstrated in both *in vitro* and *in vivo* assays (Waxman *et al*, 2001), the effects of geldanamycin on the replication of HCV RNA have not yet been examined in replicon cells. The HCV replicon cell line used in the present study does not contain an NS2-coding region, and NS2 has been shown to be unnecessary for the replication of HCV subgenomic replicon (Lohmann *et al*, 1999). Therefore, the observed reduction in RNA replication in the HCV replicon cells by treatment with geldanamycin was not due to an inhibition of HCV polyprotein processing. *In vitro* pull-down assays revealed that geldanamycin inhibited the binding of FKBP8 to Hsp90 and/or NS5A domain I (Supplementary Figure 4). Thus, geldanamycin may inhibit HCV replication by disruption of NS5A/FKBP8/Hsp90 complex. These results suggest that a protein complex composed of FKBP8, Hsp90, and NS5A is involved in HCV RNA replication.

Discussion

HCV NS5A is a multifunctional protein involved in viral replication and pathogenesis (Macdonald and Harris, 2004). In this study, we demonstrated that NS5A specifically binds to FKBP8, but not to other homologous immunophilins such as FKBP52 and CypD, and that FKBP8 forms both a homomultimer and a heteromultimer with Hsp90. Mutation analyses of FKBP8 and Hsp90 suggest that FKBP8 intermediates between NS5A and Hsp90 via the different position in the TRP domain. FKBP8 has been shown to be expressed in several human tissues, including the liver (Lam *et al*, 1995); moreover, it has been demonstrated that FKBP8-knockout mice exhibit unusual morphological changes in brain development in the embryonic stage (Nielsen *et al*, 2004). However, the physiological function of FKBP8 has not been clarified to date.

Recently, the *in vitro* replication of the full-length HCV genome of genotype 2a (JFH1) isolated from an HCV-infected patient who developed fulminant hepatitis was reported (Lindenbach *et al*, 2005; Wakita *et al*, 2005; Zhong *et al*, 2005). Although binding of NS5A of the JFH1 clone to FKBP8 was weaker than that of genotypes 1a and 1b (Figure 2A), siRNA-mediated knockdown of FKBP8 impaired production of infectious HCV particles in JFH1 cell culture system (Figure 6D). In spite of a weaker interaction between FKBP8 and NS5A, these results suggest that FKBP8 is still required for HCV replication in the cell culture system of JFH1. The involvement of FKBP8 in mitochondria-mediated apoptosis remains controversial. Shirane and Nakayama (2003) reported that FKBP8 binds to Bcl-2 and that the Bcl-2/FKBP8 complex was sequestered in the mitochondria in order to suppress apoptosis. However, Edlich *et al* (2005) reported that FKBP8 binds to calmodulin via elevations in the calcium concentration, which in turn leads to the promotion of apoptosis in neuronal tissues. Knockdown of FKBP8 led to impaired HCV RNA replication, which was restored by the expression of an RNAi-resistant FKBP8 mutant. These results suggest that the impairment of HCV RNA replication induced by the knockdown of FKBP8 was not due to an induction of apoptosis, nor to any side effects of RNA transfection. The modulation of apoptosis by FKBP8 might be diverse in different tissue types and cell lines.

FKBP8 belongs to the FKBP family due to sequence similarity, but neither FK506 binding nor PPIase activity has been detected in the case of FKBP8 thus far (Lam *et al*, 1995). Apoptosis was induced in the SH-SY5Y neuroblastoma cell line by the treatment with mitochondria-mediated proapoptotic drugs, but was inhibited by the knockdown of FKBP8 and was enhanced by treatment with GPI1046, a nonimmunosuppressive FK506 derivative, whereas this result was not obtained with FK506 (Edlich *et al*, 2005). The inhibition constant of FKBP8 to FK506 was 50 times higher than that of FKBP12 to FK506 (Edlich *et al*, 2005), which suggests that the binding affinity of FKBP8 to FK506 is low. Furthermore, cyclosporin A, but not FK506, was shown to suppress HCV RNA replication via the interaction of NS5B with CypB